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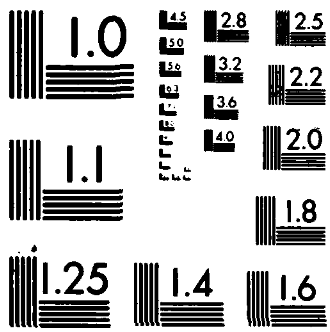

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MEASUREMENT OF THE DISTRIBUTION OF INDIUM-111 ON HUMAN PLASMA PROTEINS USING CHROMATOGRAPHY AND IMMUNOPRECIPITATION

by

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The distribution of radioactivity on plasma proteins labeled by addition of Indium-111-oxine to citrated plasma was investigated. Analysis of plasma proteins separated on Sephadex G-200 columns showed that 23-36% of the In-111 was associated with proteins with molecular weight greater than 200,000 daltons and the remaining In-111 was associated with proteins with molecular weight less than 100,000 daltons, presumably transferrin. Affinity chromatography experiments showed that less than 2% of the radioactivity was associated with albumin. Further identification of the labeled proteins and quantitation of

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associated radioactivity was performed by precipitating specific proteins with antibody. These studies showed that the In-111 was distributed on transferrin (54-76%), fibrinogen (11-24%), IgM (8-20%), C3 (10-21%), and haptoglobin (3-8%). In-111 associated with fibrinogen, IgM, and haptoglobin was overestimated in some experiments due to binding of labeled C3 to the antigen-antibody precipitates.

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ABSTRACT

The distribution of radioactivity on plasma proteins labeled by addition of Indium-111-oxine to citrated plasma was investigated. Analyses of plasma proteins separated on Sephadex G-200 columns showed that 23-36% of the In-111 was associated with proteins with molecular weight greater than 200,000 daltons and the remaining In-111 was associated with proteins with molecular weight less than 100,000 daltons, presumably transferrin. Affinity chromatography experiments showed that less than 2% of the radioactivity was associated with albumin. Further identification of the labeled proteins and quantitation of associated radioactivity was performed by precipitating specific proteins with antibodies. These studies showed that the In-111 was distributed on transferrin (54-76%), fibrinogen (11-24%), IgM (8-20%), C3 (10-21%), and haptoglobin (3-8%). In-111 associated with fibrinogen, IgM, and haptoglobin was overestimated in some experiments due to binding of In-111 labeled C3 to the antigen-antibody precipitates.

INTRODUCTION

Indium-111-oxine is an effective gamma-emitting agent for in vitro labeling of platelets and leukocytes (Thakur et al, 1976, 1977) which can be used for scintigraphic imaging of venous thrombus and abscess. During the labeling procedure, the presence of plasma is one of the most important factors influencing the uptake of In-111-oxine by the platelets and leukocytes. Binding of In-111 by plasma transferrin is believed to interfere with the labeling of cells. Using electrophoresis, Thakur et al (1977) showed that approximately 90% of the In-111 in plasma migrates in the protein band containing transferrin. However, Scheffel et al (1979) reported that addition of transferrin did not affect platelet uptake of In-111 in the absence of plasma. Thus, the In-111 labeled plasma proteins needed to be clearly identified.

In the present study, chromatography and immunoprecipitation procedures were used to identify the plasma proteins labeled with In-111. In initial experiments, the plasma proteins were separated according to molecular weight using Sephadex G-200 column chromatography, and the In-111 in the eluted fractions was measured. These results suggested that, in addition to transferrin, fibrinogen and other proteins were labeled. We identified the individual proteins that bind In-111 by using antibodies to precipitate specific proteins.

METHODS

Human plasma was obtained from fresh whole blood collected in ACD anticoagulant (NIH, Formula A) by centrifugation at 1500 X g for 5 minutes. The plasma was removed and centrifuged a second time to remove any residual cells. The plasma was then labeled by adding 10-150 microcuries of Indium-111-oxine* to 10-20 ml plasma. A minimum time of 30 minutes at room temperature was allowed for labeling to occur before further testing. For procedures in which serum rather than plasma was analyzed, the In-111-labeled plasma was clotted by incubation with thrombin at 37 C for 15-30 minutes.

Gel filtration experiments were performed with a Sephadex G-200 column (1.5 X 75 cm) equilibrated with 0.1 M Tris in 0.2 M NaCl (pH 8.0) (Flodin and Killander, 1962). A 2 ml sample of In-111-labeled plasma or serum was applied to the top of the column. Elution of protein from the column was carried out with the same buffer at a flow rate of 20-30 ml/hr and effluent fractions were collected at 5 or 10 minute intervals using a fraction collector operating on a time basis. Radioactivity in each fraction was measured in a gamma counter, and protein concentration was estimated by absorbance at 280 nm in a spectrophotometer.

Affinity column chromatography was performed to demonstrate the presence or absence of radioactivity on the albumin portion of plasma proteins. A 1.0 ml sample of plasma was applied to a small column

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*Mediphysics, Inc., Emeryville, CA

containing 6.0 ml of CM Affi-gel Blue† according to the manufacturer's directions. The plasma proteins, except for albumin which adhered to the column, were eluted with 3 volumes of 0.1 M K_2PO_4 in 0.15 M NaCl, pH 7.25 (buffer). Then the albumin was eluted using 3 volumes of 1.4 M NaCl in the buffer. During the elution procedure, effluent fractions of 1-2 ml were collected for quantitation of radioactivity and estimation of protein concentration.

Analysis of specific proteins for presence of In-111 radioactivity was performed using an immunoprecipitation technique. Antibodies were used to precipitate specific proteins from solution in order to test for protein-associated radioactivity. The antibodies used were IgG fractions‡ of goat antibodies to human protein with specificities for the following antigens: fibronectin, fibrinogen, α-2-macroglobulin, IgM, IgA, IgG, transferrin, haptoglobin, C3, C4, C5, and plasminogen. Prior to testing with antibodies, the In-111-plasma was refrigerated overnight at 4 C and then centrifuged at 5000 X g for 5 minutes to remove any precipitable protein. The amount of radioactivity (cpm/ml) in the plasma was not altered by this procedure. Then, 0.20 ml of plasma was added to 0.2 ml of antibody in a polystyrene centrifuge tube, with the exception of the fibrinogen antibody and transferrin antibody tubes to which 0.30 ml of antibody was added. Antibody control tubes contained 0.20 ml of antibody and 0.20 ml of saline; plasma control tubes contained 0.20 ml of plasma and 0.20 ml of saline. The tubes were mixed,

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†Bio-Rad Laboratories, Richmond, CA

‡Cappel Laboratories, Inc., Cochranville, PA

incubated at 37 C for one hour, and refrigerated overnight at 4 C. The tubes were then centrifuged at 5000 X g for 5 minutes, the supernatant solution was saved, and the precipitated protein was washed four times with 0.5 ml of cold saline, centrifuging each time at 5000 X g for 5 minutes. The tubes were maintained at 4 C throughout this procedure. The radioactivity in the precipitated protein, supernatant solutions, and wash solutions for each tube was measured in a gamma counter. The radioactivity in each antigen-antibody precipitate was corrected by subtracting the radioactivity precipitated in the plasma control tube without antibody. The percent of In-111 associated with each specific protein was then determined by dividing the cpm in the precipitated protein by the total radioactivity in the precipitate, supernatant solution, and wash solution. The supernatant solution was analyzed for the presence of excess antibody or excess antigen by further testing against plasma or antibody in agarose gel diffusion plates.

After evaluation of results suggested that In-111 was bound to C3, additional studies were performed to determine whether some of the radioactivity in the immunoprecipitates was due to complement binding. In one experiment, the amount of C3 remaining in the supernatant solution was estimated by precipitation with C3 antibody. The amount of radioactivity precipitated by C3 antibody was compared with that of the plasma control in order to calculate the portion of C3 remaining in the supernatant solution. This was done to avoid misinterpretation of the results when In-111 labeled C3 was bound to the precipitated antigen-antibody complex. In another experiment, C3 protein was removed from

the In-111 labeled plasma by precipitation with C3 antibody prior to testing with the other specific antibodies. A 0.20 ml volume of C3 antibody was added to 0.20 ml of In-111 plasma in each of the 12 tubes and tested as previously described. The supernatant solution from each tube was saved, then 0.20 ml of supernatant solution was added to 0.20 ml of each of the specific antibodies. These tubes were incubated, refrigerated, washed, and counted as previously described. The percent of In-111 radioactivity associated with each protein was calculated.

RESULTS

Column chromatography with Sephadex G-200 separated the plasma proteins into three major portions according to their molecular weight. The In-111 radioactivity emerged from the column in two peaks, one associated with the first peak of protein and the other with the third peak of protein. Fig. 1 shows a typical pattern from one of five experiments. In initial experiments, the presence of proteins of known molecular weight in each peak was confirmed by reactivity of the eluate with antibodies in immunodiffusion plates. The first peak of eluted protein contained high molecular weight proteins (> 200,000 MW) consisting of fibronectin, fibrinogen, α-2-macroglobulin, and IgM. In five experiments on human plasma, the amount of radioactivity associated with this first peak ranged from 23-36% of the total radioactivity eluted from the column. The second peak of protein contained proteins of approximately 150,000 daltons, mainly IgG and IgA. There was no apparent In-111 radioactivity associated with the second peak of protein in any of the experiments. The third peak of protein, which was predominantly albumin (66,000 MW) and transferrin (77,000 MW), contained the most radioactivity, ranging from 62-77%.

Additional chromatography experiments were performed in which the original In-111 plasma was clotted and the serum was applied to the column. The elution pattern of the serum sample was compared with that of the original plasma sample. Fig. 2 shows the results of the serum sample that corresponds to the plasma sample in Fig. 1. The percent of

In-111 radioactivity associated with the high molecular weight proteins was reduced by clotting from 33% in the plasma sample to 4% in the serum sample. In three experiments comparing plasma and serum, the radioactivity associated with the high molecular weight proteins was reduced by 77-88% by clotting.

Affinity chromatography experiments were performed to determine whether In-111 was associated with albumin. Affi-Gel Blue was used to selectively adsorb albumin from the plasma sample. Results from one of two experiments are shown in Fig. 3. Almost all the In-111 radioactivity (> 98%) passed through the affinity column. When albumin was eluted, it contained only 1-2% of the total radioactivity, indicating that very little In-111 radioactivity was associated with albumin.

To further identify the proteins that were labeled with In-111, antibodies were used to precipitate specific proteins to measure the associated radioactivity. These experiments were done on four different occasions and showed that certain proteins appeared to be labeled consistently (Table 1). Transferrin showed the highest percentage of associated radioactivity. The high molecular weight proteins that were labeled were fibrinogen and IgM, and there was also In-111 radioactivity associated with C3 and haptoglobin.

There was concern that In-111 labeled C3 could bind to antigen-antibody complexes and cause an overestimation of the In-111 associated with other proteins. Therefore, in one experiment, after precipitation of the specific proteins with antibody, the portion of C3 remaining in the supernatant solution was estimated. Table 2 shows that there was a

significant percentage of In-111 associated with fibrinogen, IgM, transferrin, haptoglobin, and C3. It also shows that there was considerable binding of C3 by the antibodies to fibrinogen, IgM, and haptoglobin, as there was less than half of the original C3 remaining in the supernatant solution. It should also be noted that the amount of In-111 associated with transferrin was underestimated because of antigen excess during testing.

In another experiment on the same In-111 plasma, C3 was first removed by immunoprecipitation and then the supernatant plasma was tested with specific antibodies (Table 3). These results more accurately reflect the distribution of In-111 in this sample of plasma. Comparison of results in Table 3 with those in Table 2 shows that the percent of In-111 associated with the precipitated fibrinogen, IgM, and haptoglobin was lower when C3 was not present. In the fourth column of Table 3, the percent of In-111 associated with each protein has been corrected for the absence of C3 in the sample tested.

DISCUSSION

These chromatography and immunoprecipitation experiments have confirmed that transferrin is the major plasma protein that binds In-111. Our estimates of the In-111 associated with transferrin ranged from 59-77%. This is somewhat lower than the value of 90% reported by Thakur et al (1977) using an electrophoretic technique.

The column chromatography experiments showed that In-111 was also associated with proteins of high molecular weight. The labeling of fibrinogen was suggested by the reduction in radioactivity associated with high molecular weight proteins when the In-111 plasma was clotted. Further experiments using immunoprecipitation confirmed that In-111 was bound to fibrinogen and transferrin and demonstrated In-111 labeling of IgM, C3, and haptoglobin.

The percent of In-111 associated with different proteins cannot be measured with great precision by immunoprecipitation but the proteins can be clearly identified. We tested the proteins which were present in the highest concentration in plasma. Albumin was not studied in the immunoprecipitation tests because of its very high concentration in plasma and because the antisera did not precipitate albumin in test tubes. Affinity chromatography experiments showed that less than 2% of the total radioactivity was associated with albumin.

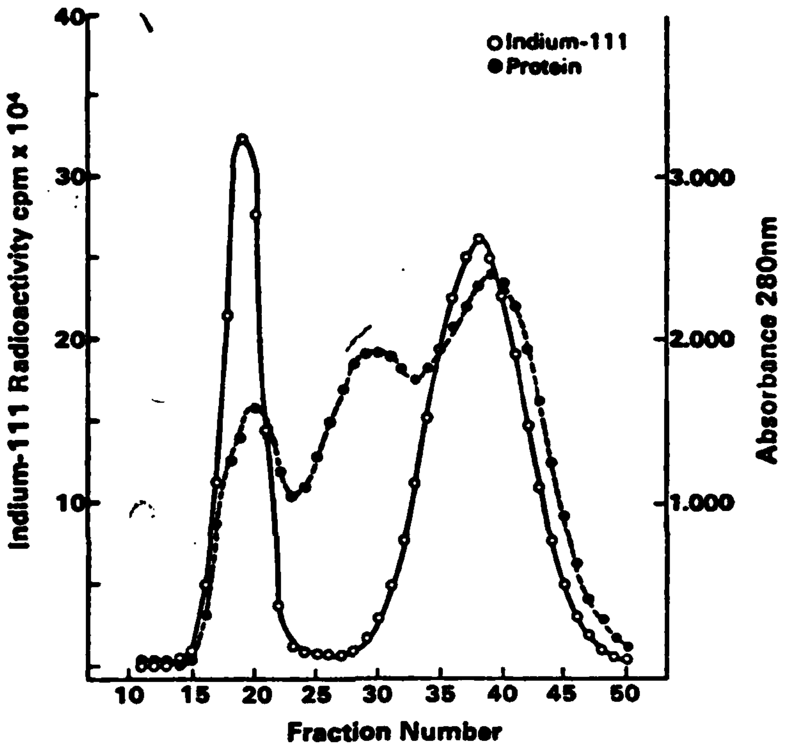
The immunoprecipitation experiments overestimated the amount of In-111 associated with fibrinogen, IgM, and haptoglobin due to binding of radiolabeled complement to the antigen-antibody complexes. Attempts

to inactivate complement by heating at 56 C for 30 minutes resulted in precipitation of fibrinogen from the plasma. In one experiment, C3 was successfully removed from the plasma by precipitation with C3 antibody prior to testing with the other specific antibodies. Comparison of results before and after removal of C3 showed that the removal of C3 was associated with reduced values for In-111 labeled fibrinogen, IgM, and haptoglobin.

The finding of In-111 associated with fibrinogen, IgM, C3, and haptoglobin was unexpected. None of these proteins is known to have a natural affinity for heavy metals as does transferrin. Other investigators have labeled fibrinogen with In-111 by using bifunctional chelators (Goodwin et al, 1976) and with Tc-99 by using reductive electrolysis (Harwig et al, 1976). The ability to label fibrinogen with In-111-oxine could have clinical applications if the binding is sufficiently stable and the protein retains its function in vivo.

FIGURE 1

Indium-111 radioactivity associated with fractions of plasma proteins separated on Sephadex G-200. One of the five experiments performed is reported.

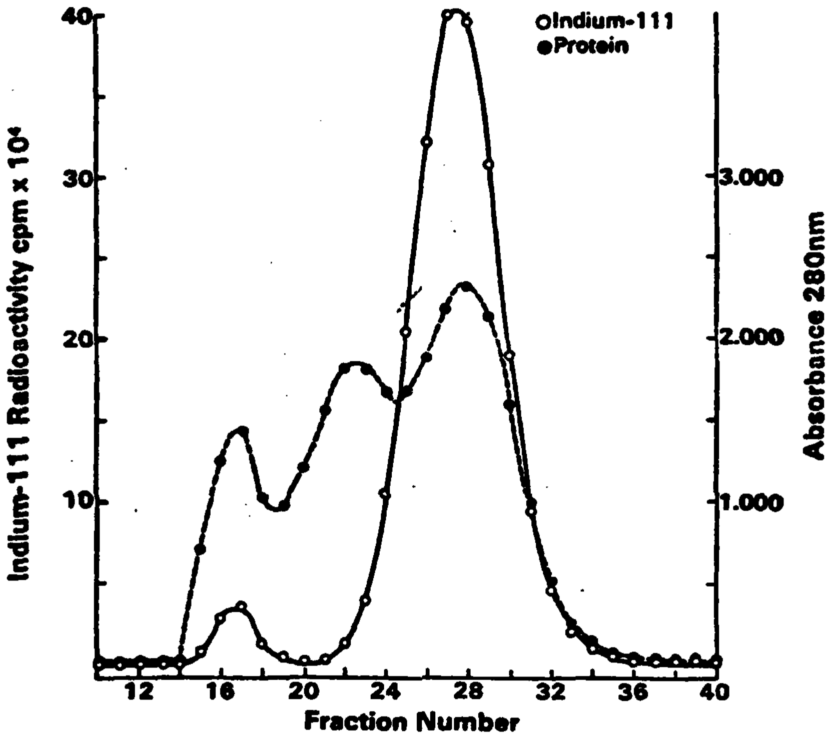


Indium-111
Protein
Indium-111 Radioactivity cpm x 10⁴
Absorbance 280nm
Fraction Number
40
30
20
10
3.000
2.000
1.000
10
15
20
25
30
35
40
45
50

FIGURE 1

FIGURE 2

Indium-111 radioactivity associated with fractions of serum proteins separated on Sephadex G-200. One of the three experiments performed is reported. The serum studied was a clotted sample of the same In-111 plasma shown in Fig. 1.

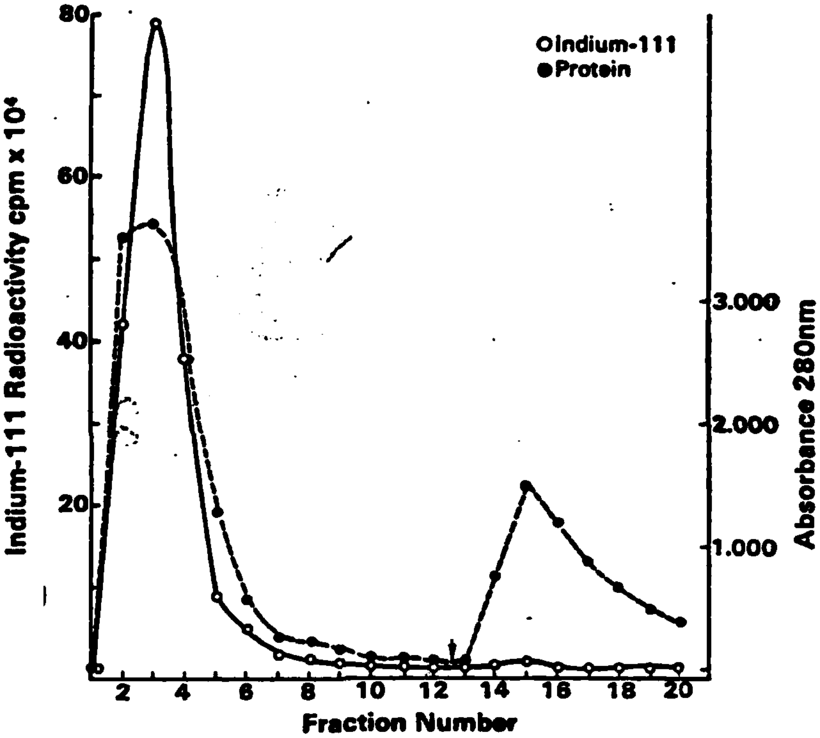


OIndium-111
●Protein
Indium-111 Radioactivity cpm x 10⁴
Absorbance 280nm
Fraction Number
40
30
20
10
3.000
2.000
1.000
12
16
20
24
28
32
36
40

FIGURE 2

FIGURE 3

Indium-111 radioactivity associated with fractions of plasma proteins eluted from Cm-Affi-Gel blue albumin affinity column for one of the two experiments performed. Arrow indicates the beginning of elution of bound albumin.



80
60
40
20
2
4
6
8
10
12
14
16
18
20
Fraction Number
Indium-111 Radioactivity cpm x 10⁴
Absorbance 280nm
3.000
2.000
1.000
OIndium-111
●Protein

FIGURE 3

TABLE 1

PERCENT OF In-111 ASSOCIATED WITH PROTEINS PRECIPITATED FROM PLASMA BY SPECIFIC ANTIBODIES IN FOUR EXPERIMENTS

Protein	Experiment 1	Experiment 2	Experiment 3	Experiment 4
Fibronectin	3	0	4	1
Fibrinogen	11	24	19	24
α-2-macroglobulin	4	0	0	0
IgM	8	18	NT*	20
IgA	0	4	NT	0
IgG	0	0	NT	0
Transferrin	67	59	76	54
Haptoglobin	4	6	3	8
Plasminogen	0	0	2	0
C3	NT	20	21	10
C4	NT	0	9	0
C5	NT	5	18	2

*NT means not tested

TABLE 2

IMMUNOPRECIPITATION OF PROTEINS FROM In-111 PLASMA

Protein	Precipitate cpm X 10^{-3}	Total cpm X 10^{-3}	Percent In-111 Precipitated	Percent Supernatant C3 Remaining
Fibronectin	3	255	1	60
Fibrinogen	66	280	24	10
α-2-macroglobulin	<1	280	0	70
IgM	57	280	20	10
IgA	1	280	0	80
IgG	<1	276	0	80
Transferrin	152	282	54*	100
Haptoglobin	22	285	8	50
Plasminogen	<1	275	0	90
C3	27	272	10	0
C4	2	283	1	80
C5	6	287	2	60

*Underestimation due to antigen excess during testing

TABLE 3

IMMUNOPRECIPITATION OF PROTEINS FROM In-111 PLASMA AFTER REMOVAL OF C3

Protein	Precipitate cpm X 10^{-3}	Total cpm X 10^{-3}	Percent In-111 Precipitated	Percent of Total Protein In-111
Fibronectin	1	179	<1	<1
Fibrinogen	32	181	18	16
α-2-macroglobulin	2	187	1	<1
IgM	20	178	11	10
IgA	1	182	<1	<1
IgG	1	186	<1	<1
Transferrin	151	185	82	74
Haptoglobin	7	175	4	4
Plasminogen	1	184	<1	<1
C3	1	185	<1	10
C4	4	180	2	2
C5	2	181	1	<1

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